Fluoroquinolones (FQs) are some of the most frequently used antimicrobials worldwide for the treatment of both gram-negative and gram-positive bacterial infections (2). The World Health Organization (WHO) has classified quinolones and FQs as “critically important antimicrobials” because of their broad-spectrum effects and clinical importance in both human and animal medicine (40). Because the importance of FQs in humans and animals is increasing, FQ-resistant bacteria are a major concern in the treatment of infectious diseases.

Antimicrobials are widely used in the food-producing animal industry; they are used as feed additives to promote growth and prevent disease and also for therapeutic purposes (20). On average, antimicrobial use is higher in the animal industry than in human medicine (39). The swine industry is the most important livestock industry in South Korea, and the consumption of antimicrobials in the swine industry has been estimated to represent approximately 60% of all antimicrobial consumption in the animal industry in Korea (23). Consequently, the antimicrobial-resistant rate in swine is relatively higher than that in other food-producing animals in Korea (4, 22).

In an effort to reduce antimicrobial resistance in food-producing animals, many antimicrobial agents, including FQs, have been prohibited for use in feed additives since 2009 in Korea (4). Consequently, FQ-resistant bacteria were expected to decrease in the animal industry in Korea. Accordingly, in this study, we investigated the prevalence of FQ-resistant Escherichia coli isolated from swine feces. In addition, we characterized three major FQ resistance mechanisms in FQ-resistant E. coli: (i) target-enzyme modification (mutations in quinolone resistance–determining regions [QRDRs]), (ii) plasmid-mediated quinolone resistance gene (PMQR) activity, and (iii) extrusion of drug agents by efflux pump activity (43).

**MATERIALS AND METHODS**

**Isolation of E. coli.** A total of 237 swine fecal samples were collected from 24 swine farms in Korea (Jeju province, n = 86; Jeolla province, n = 82; Gyeongsang province, n = 35; Chungcheong province, n = 24; Gyeonggi province, n = 6; Gangwon province, n = 4) from March to June 2015. Of the 237 samples, 53 (22.4%) were from swine with diarrhea. The other 184
samples (77.6%) were collected from healthy swine. All fecal samples were streaked on 5% sheep blood containing Columbia agar (bioMérieux SA, Marcy l’Etoile, France) (6), and candidate E. coli colonies were inoculated on MacConkey agar plates (BD, Sparks, MD) and triple sugar iron agar (BD) (29). These inoculated plates were incubated at 37°C for 24 h. PCR targeting the 16S ribosomal RNA region was performed to identify E. coli, as described previously (33). Only one E. coli isolate per sample was selected for further analysis.

Identification and antimicrobial susceptibility tests of FQ-resistant E. coli. For all isolated E. coli, standard disk diffusion tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline (38) to identify FQ-resistant E. coli isolates and to determine their antimicrobial resistance profiles. Antimicrobial susceptibility tests were performed using the following antimicrobial disks (BD): ampicillin (10 µg), amoxicillin–clavulanic acid (20/10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefotetan (30 µg), ceftazidime (30 µg), imipenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (CIP; 5 µg), nalidixic acid (NA; 30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), and chloramphenicol (30 µg). The tests were performed according to the CLSI guideline (38). E. coli ATCC 25922 was used as a reference strain.

MICs of NA and CIP. MICs of NA and CIP for FQ-resistant E. coli were determined by the broth microdilution method according to the CLSI guideline (38). The concentrations of these two antimicrobial agents were 0.008 to 1.024 µg/mL. E. coli ATCC 25922 was used as a reference strain.

Comparison of MDR and ESBL-producing E. coli rates in low and high CIP MIC groups. To analyze the relationship between the levels of CIP MIC and MDR and ESBL-producing E. coli, FQ-resistant isolates were divided in two groups based on MICs, similar to a study by Karlowsky et al. (18): the high MIC group (MICs of ≤16 µg/mL) and the low MIC group (MICs of >16 µg/mL). The frequencies of MDR and ESBL-producing E. coli in the two groups were compared.

Identification of mutations in QRDRs and detection of PMQRs. DNA samples for PCR amplification were prepared from all FQ-resistant E. coli using a standard heat lysis protocol (42). All PCR primers used in this study are listed in Table 1. For detection of gene mutations, each QRDR (gyrA, gyrB, parC, and parE) was first amplified by PCR, and the PCR fragments were custom sequenced by Biofact Co. (Seoul, Korea). Mutations in QRDRs were identified by comparing the sequencing data with those of the E. coli K-12 strain (GenBank accession no. U00096). PMQR genes (qnrA, qnrB, qnrS, qepA, and aac(6’)-Ib-cr) were detected by PCR amplification and sequencing analysis, as described in previous studies (9, 25, 41, 42).

OST test. Organic solvent tolerance (OST) tests were carried out to identify phenotypic efflux pump activity (37). A single colony was inoculated in 2 mL of Luria-Bertani broth for 5.5 h to reach the logarithmic phase. Cultures in the logarithmic phase were

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli identification</td>
<td>E. coli 16S rRNA</td>
<td>F: GGGAGTAAAGTTAATACCTTTGCTC R1: TTCCGGAAGGCACATTCT R2: TTCCGGAAGGCACCAATC</td>
<td>584</td>
</tr>
<tr>
<td>QRDR</td>
<td>gyrA</td>
<td>F: TACACCCGTCACACATTTGAGG R: TTAATGTATGCGCCCGTCGG</td>
<td>648</td>
</tr>
<tr>
<td></td>
<td>gyrB</td>
<td>F: GAAATGACCCGCGCCTTGAATG R: ACGCAGTATCCACAGGCC</td>
<td>272</td>
</tr>
<tr>
<td></td>
<td>parC</td>
<td>F: AAACCTGTTCACGCGCCTATTAG R: GTCGTTCCGTTAAGCAGAA</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>parE</td>
<td>F: CTGACCGGAAGCTAGTCGAACC R: CGTTCGGCTGCTTTTCGG</td>
<td>892</td>
</tr>
<tr>
<td>PMQR</td>
<td>qnrA</td>
<td>F: CAACCTTGAGTGCCCAATGCG R: GACTCTTTCGAGTTGACCC</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>qnrB</td>
<td>F: GGMATHGAAATTCCGCGAATCGT R: TTTGCAGGYYCGCGATC</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>qnrS</td>
<td>F: ACGACATTCGTCACGTAATCAGA</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td>qepA</td>
<td>F: GCAGGTCACGCGAGGTTAG R: CTTCCTGGCAGGATACGTG</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>aac(6’)-Ib</td>
<td>F: TTGCCGATGCTCCTAGTAGTTAGTA R: CTCGAATGCCTGGCGTG</td>
<td>482</td>
</tr>
</tbody>
</table>

TABLE 1. Sequences of primers
diluted to an optical density of 0.2 (530 nm). Next, 100 μL of diluted culture was spread on Luria-Bertani agar, and 10 mL of an organic solvent (hexane-cyclohexane [3:1]) was overlaid. The plates were sealed with parafilm to prevent evaporation and were incubated at 30°C overnight. The following day, the colony numbers were counted, and the results were analyzed as follows: strong efflux pump activity (++) ≥100 colonies; weak efflux pump activity (+) 1 to 99 colonies; no efflux pump activity (−), no growth. The ATCC 25922 strain was included as a reference strain.

Statistical analysis. All statistical analyses were performed using SPSS software version 23 (SPSS Inc., IBM Corp., Armonk, NY). Chi-square tests were used for comparison of the frequencies of FQ-resistant isolates in E. coli from healthy and diseased animals and for comparisons of the frequencies of MDR and ESBL-producing E. coli in low and high CIP MIC groups. Differences with P values of less than 0.05 were considered significant. The correlations between the number of mutations in QRDRs or the OST activity and CIP MICs were analyzed by Pearson’s correlation coefficient tests (21).

RESULTS

Isolation of FQ-resistant E. coli and antimicrobial susceptibility tests. E. coli was isolated from 171 (72.2%) of 237 swine fecal samples. Among the 171 E. coli isolates, 52 (30.4%) were from diseased swine, and the other 119 (69.6%) were from healthy swine. Of the 171 isolates, 59 (34.5%) were confirmed as FQ-resistant E. coli, among which 21 (40.4%) of 52 were from diseased swine and 38 (31.9%) of 119 were from healthy swine. Differences in the frequencies of FQ-resistant isolates in E. coli from diseased and nondiseased pigs were not statistically significant (P > 0.05). Of 59 FQ-resistant E. coli, 54 (91.5%) isolates were classified as MDR (Table 2). The rates of resistance to seven different classes of drugs are described in Table 2. Eleven isolates (18.6%) were confirmed as ESBL-producing E. coli. FQ-resistant isolates had MIC values against CIP ranging from 4 to 256 μg/mL, and all of these isolates were phenotypically resistant to NA (256 μg/mL and >1,024 μg/mL; Table 3). When FQ-resistant isolates were divided into two groups based on CIP MICs, the frequencies of both MDR and ESBL-producing E. coli in the high MIC group were significantly higher than those in the low MIC group (Table 4).

Presence of amino acid substitutions in QRDRs in FQ-resistant E. coli. DNA sequences were analyzed to target the QRDRs for DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE). All 59 isolates had at least one site of QRDR amino acid substitutions in gyrA, parC, and parE. There were no gyrB gene mutations in any of the isolates in this study. One isolate (1.7%) had a single mutation in gyrA, 36 isolates (61.0%) had mutations in both gyrA and parE, and 22 isolates (37.3%) had mutations in gyrA, parC, and parE (Table 3). The gyrA amino acid substitutions were Ser83Leu (100%), Asp87Asn (69.5%), Asp87Gly (11.9%), Asp87Tyr (11.9%), and Asp87His (3.4%), and the parC mutations were Ala56Thr (8.6%), Ser80Ile (96.6%), Ser80Arg (1.7%), Glu84Gly (12.1%), Glu84A la (8.6%), and Glu84Lys (1.7%). In parE, Ile355Thr
(22.7%), Leu416Phe (4.5%), Ser458Ala (54.5%), Glu460Lys (13.6%), and Ile464Phe (9.1%) were observed. Notably, all FQ-resistant E. coli, except two isolates with lower CIP MICs, had a double mutation (Ser83Leu and amino acid substitution in aspartic acid 87) in gyrA, with additional mutations in parC and/or parE. The correlation between the total numbers of mutations in QRDRs and the CIP MIC was shown to be moderate, as measured by Pearson’s correlation coefficient (R value: 0.546; data not shown).

Detection of PMQR genes in FQ-resistant E. coli. Nine (15.3%) of 59 FQ-resistant isolates harbored PMQR genes (qnrS, qepA, and aac(6’)-Ib-cr), whereas none of these isolates had qnrA or qnrB genes. Each of the nine isolates harbored only one of the PMQR genes screened. The predominant PMQR gene detected in this study was qnrS (seven isolates), whereas qepA and aac(6’)-Ib-cr were found in only one isolate each.

Phenotypic efflux pump activity. Phenotypic efflux pump activity was measured by OST tests (37). Most (56, 94.9%) of the FQ-resistant E. coli isolates showed efflux pump activity against organic solvent. Among the isolates with positive efflux pump activity, 40 (67.8%) were found to have strong efflux pump activity, and 16 (27.1%) had moderate activity. Notably, the CIP MIC was not correlated with phenotypic efflux pump activity (R value: 0.044; data not shown).

DISCUSSION

FQ has been prohibited as a feed additive since 2009 in an effort to reduce antimicrobial resistance in food-

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TABLE 3. Amino acid substitutions in the QRDRs and prevalence of PMQR genes for different CIP MICs in 59 FQ-resistant E. coli isolates

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>gyrA</th>
<th>parC</th>
<th>parE</th>
<th>Isolate no.</th>
<th>PMQR (isolate no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>CIP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.008</td>
<td>S83L</td>
<td>S80I</td>
<td>ATCC 25922</td>
<td>qnrS (58)</td>
</tr>
<tr>
<td>256</td>
<td>4</td>
<td>S83L</td>
<td>S80I</td>
<td>1</td>
<td>qnrS (1)</td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>8</td>
<td>S83L/D87N</td>
<td>S80I</td>
<td>4, 5, 26, 30, 31, 37, 46, 53, 59</td>
<td></td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>16</td>
<td>S83L/D87G</td>
<td>S80I</td>
<td>15, 16, 18, 19, 43, 45</td>
<td></td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>32</td>
<td>S83L/D87N</td>
<td>S80I</td>
<td>22, 44</td>
<td></td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>64</td>
<td>S83L/D87N</td>
<td>S80I</td>
<td>56, 57</td>
<td></td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>128</td>
<td>S83L/D87N</td>
<td>S80I</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>&gt;1,024</td>
<td>256</td>
<td>S83L/D87N</td>
<td>S80I</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Total no. of isolates</td>
<td>59 (11)</td>
<td>9 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a ESBL-producing E. coli.
b The numbers in parentheses indicate the total number of ESBL-producing E. coli isolates in each case.

TABLE 4. Proportions of MDR and ESBL-producing E. coli in the low and high CIP MIC groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CIP MIC (µg/mL)</th>
<th>No. of MDR</th>
<th>No. of ESBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low MIC group, n (%)</td>
<td>28</td>
<td>≤16</td>
<td>23 (82.1)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>High MIC group, n (%)</td>
<td>31</td>
<td>&gt;16</td>
<td>31 (100)</td>
<td>10 (32.3)</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td></td>
<td>54</td>
<td>11</td>
</tr>
</tbody>
</table>

Note that the frequencies of MDR and ESBL-producing E. coli were statistically different in the low and high MIC groups.
producing animals in Korea (4). However, the current study indicated that FQ resistance in *E. coli* (34.5%) isolated from pigs from Korea was higher than that from other countries (27, 34) and has significantly increased within the last decade (8.0% in 2004) (22). This is largely due to the massive use of FQ in livestock for therapeutic and self-treatment purposes, instead of as feed additives; thus, the total amounts of FQ did not change substantially before and after the ban of FQ as a feed additive (the total amount of FQ used in livestock in 2008 was 51,257 kg, and that in 2015 was 49,161 kg) (4). Therefore, additional regulations reducing the use of FQ in food-producing animals may be needed to reduce FQ resistance in Korea.

To date, three mechanisms through which bacteria acquire FQ resistance have been reported. One of the most important resistance mechanisms is drug target modifications in DNA gyrase and topoisomerase IV. Gyrase is encoded by *gyrA* and *gyrB*, and topoisomerase IV is encoded by *parC* and *parE* (16). Because DNA gyrase is the primary target of FQ in gram-negative bacteria, *gyrA* mutations are dominant mutations in *E. coli* (7). Consequently, in this study, all 59 isolates (100%) had *gyrA* mutations, and most of them (57 of 59, 96.6%) were double amino acid substitutions (Ser83Leu plus substitution in aspartic acid 87). These results were consistent with previous studies that suggested that double mutations in *gyrA* were closely related to FQ resistance in *E. coli* (14, 17, 19). With the exception of one isolate that had a single mutation in *gyrA*, all mutants that had *parC* and *parE* mutations also had double mutations in *gyrA*. These data supported previous studies showing that *gyrA* mutations are a prerequisite for subsequent mutations in gram-negative bacteria (15, 43) and that mutations in the *parC* and/or *parE* gene are closely related to double mutations in the *gyrA* gene (8, 19). Among *gyrA* mutant isolates, the Ser83Leu (59 of 59, 100%) amino acid substitution was the most frequent mutation, whereas that in *parC* mutants was the Ser80Ile (56 of 58 isolates, 96.6%) mutation. This result is consistent with that observed in *E. coli* isolates from humans (10, 35). In terms of the CIP MIC, the total number of point mutations in QRDR was positively correlated with the increased MIC.

FQ-resistant *E. coli* tolerates the drug through the production of plasmid-mediated genes. The possibility of conjugation with FQ-susceptible bacteria emphasizes the importance of PMQR genes (36). To date, PMQR genes have been identified as the *qnr* family, which protects the gyrase from FQ, the modifying enzyme *aac(6’)-Ib-cr* (cr variant of *aac(6’)-Ib*), and the *qepA* gene encoding the efflux pump (43). Bacteria usually require at least double mutations in QRDRs to acquire FQ resistance (26). However, in this study, one isolate (CIP MIC: 4 μg/mL) had only a single mutation in *gyrA* and harbored *qnrS* in its plasmid. This result is consistent with the function of PMQR, contributing to FQ resistance (16).

In this study, the overall prevalence of PMQR in FQ-resistant *E. coli* from pigs was considerably higher than that in a previous study in Korea (4.3 versus 15.3%) (31). Notably, these findings indicated that the spread of PMQR genes had increased on pig farms and that the risk of PMQR spread in livestock was considerable. The prevalent PMQR gene found in this study was different from that in human isolates in Korea (32). The predominant PMQR genes detected in human isolates were *qnrA* and *qnrB*, whereas *qnrS* was the prevalent PMQR in this study, indicating that the origin of PMQR was different between humans and pigs in Korea.

We performed antimicrobial susceptibility tests against an additional seven classes of drugs (a total of 16 antimicrobial agents), including quinolone (NA) and FQ (CIP), to detect ESBL-producing *E. coli* and MDR isolates. A previous study (30) showed a high prevalence of MDR isolates in FQ-resistant bacteria. In addition, a study by Karlowsky et al. (18) found that, as the CIP MIC increases, the rates of resistance to other antimicrobials, including ampicillin, cefdinir (a third-generation cephalosporin), and nitrofurantoin, also increase. Based on these previous reports, we compared the MDR and ESBL rates in high and low CIP MIC groups (Table 4). Interestingly, all non-MDR isolates (*n* = 5, 8.5%) belonged to the low MIC group, and the rates of both MDR and ESBL-producing *E. coli* were significantly higher in the high MIC group than in the low MIC group. Although the mechanism underlying this process is still unclear, our findings suggested that FQ-resistant bacteria were strongly related to MDR and ESBL.

In our study, we did not find a correlation between CIP MICs and efflux pump activity. Of 59 CIP-resistant *E. coli*, 56 (94.9%) isolates had efflux pump activity, based on our OST results. Interestingly, isolates 4 and 5 had the same MICs, QRDR mutations, and PMQR results, but had divergent OST results. These isolates already had double mutations in *gyrA* and a single mutation in *parC*. This finding is consistent with that of a previous study showing that efflux pump activity usually does not further affect the MIC in bacteria having high levels of resistance to FQ owing to target alteration (1). Indeed, the low *R* value (0.044) found by the Pearson correlation coefficient method verified this assertion. Target modification is the most important mechanism through which *E. coli* reaches the FQ resistance breakpoint. Additionally, the presence of PMQR genes can support FQ resistance through the functions of the encoded proteins. However, when *E. coli* exhibits high levels of resistance, efflux pump activity is not an important factor.

In conclusion, the effort to reduce the use of FQ in food animals was not successful. Prohibition of FQ as a feed additive has led to alterations in FQ use for therapeutic and self-treatment purposes without substantial changes in the total amount of FQ use in the food animal industry in Korea (4). Consequently, the prevalence of FQ-resistant and MDR *E. coli* is considerably high in the Korean swine industry. Moreover, compared with a previous study in Korea (31), the current study showed that there was a considerable increase in PMQR prevalence in food-producing animals. Commensal *E. coli* from feces are often considered to be good indicators for selection pressure by antimicrobial agents and for antimicrobial resistance in a population (3, 12). The results of this study suggested that FQ resistance was high in the animal industry in Korea. Accordingly, considering the clinical importance of FQ in veterinary and human medicine, prescriptions and other uses of FQ should
be carefully monitored and regulated, in conjunction with
the ban of FQ use as a feed additive, in order to reduce FQ
resistance in food-producing animals.

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